Final Report

Original 2 of 2

Determination of 72h-EC50 of [redacted]

in *Desmodesmus subspicatus*
according to OECD 201 resp. EU C.3

Study No.: [redacted]

Sponsor:
[redacted]

Test Facility:
[redacted]

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1 GLP-Compliance Statement

It is hereby declared that all tests were made in accordance with the „Revised OECD Principles of Good Laboratory Practice" (Paris, 1997) as stated in the following guidelines:

- OECD Principles of Good Laboratory Practice, adopted by Council on 26th November 1997; Environment Directorate, Organisation for Economic Cooperation and Development, Paris 1998
- Directive 2004/10/EC of the European Parliament and of the Council of 11 February 2004 on the harmonisation of laws, regulations and administrative provisions relating to the application of the principles of good laboratory practice and the verification of their applications for tests on chemical substances (codified version)
- Chemikaliengesetz (Chemicals Act) of the Federal Republic of Germany (ChemG) §19a and §19b and annexes 1 and 2 in the version of 02 July 2008 published in Bundesgesetzblatt No. 28/2008, pp. 1146 - 1184

Responsibility for the accuracy of the information concerning the test item as well as for its authenticity rests with the sponsor.

I herewith accept responsibility for the data presented within this report.

There were no circumstances that may have affected the quality or integrity of the study.

[redacted]

[redacted]

Information on Study Organisation:

Deputy Study Director

Study Plan dated

Experimental Starting Date

Experimental Completion Date

Draft Report dated

[redacted]

2 QUALITY ASSURANCE UNIT STATEMENT

This study has been inspected by the quality assurance unit according to the principles of Good Laboratory Practice. Study Plan and Final Report were checked at the dates given below, the Study Director and the management were informed with the corresponding report.

Also, the performance of the study was inspected, and findings were reported to Study Director and management. The inspection of short-term studies (duration less than four weeks) is carried out as audit of process concerning major technical phases of at least one similar test. Frequency is once or more a quarter.

The study was conducted and the reports were written in accordance with the Study Plan and the Standard Operating Procedures of the test facility.

Deviations from the Study Plan were acknowledged and assessed by the Study Director and included in the Final Report.

The reported results reflect the raw data of the study.

Verified Procedure	Inspected on	Findings reported on	Audit report no.
Study plan	[redacted]		
Performance of study			
Draft report			
Final report			

[redacted]

Date [redacted]

Quality Assurance Manager

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3 SUMMARY

Title of Study: Determination of 72h-EC50 o[redacted]n *Desmodesmus subspicatus* according to OECD 201 resp. EU C.3

Findings and Results:

Two experiments were performed. In the first experiment the validity criteria were not met. Therefore the study was repeated.

As the test item is poorly soluble in water, a saturated solution was prepared. This was done by shaking the nominal load with the appropriate amount of deionised water for 24 hours, followed by membrane filtration. The concentrations to be tested were prepared by dilution of this solution with deionised water.

The study was performed using five concentrations ranging from 1.0 to 100 mg/L nominal concentration. The treatments were used to incubate the unicellular freshwater green alga *Desmodesmus subspicatus* for a period of 72 hours. The cell concentration of each replicate was determined by measuring the absorption at 440 nm every 24 hours with a spectral photometer. With these measured values, the number of cells was calculated (linear correlation between cell concentration and absorption given). Then the growth rate μ, the area under the growth curve (AUC[1]) and the Yield[2] were determined.

The content o[redacted]n the test solution was analytically determined using AAS. As the test item wasn't stable under test conditions, analytical measurement were performed every day of the test. Because of the [redacted]in test medium, the correlation between nominal and measured concentration was poor. Therefore, the determination of the results was based on the geometric mean of the measured concentrations.

The EC50s of potassium dichromate were tested in a current reference test. The values lay within the normal range of the laboratory.

The following results for the test item [redacted]were determined:

Endpoint	NOEC	LOEC	EC50
Growth Rate	0.69 mg/L	2.1 mg/L	13 mg/L
AUC	0.69 mg/L	2.1 mg/L	4.4 mg/L
Yield	0.69 mg/L	2.1 mg/L	2.4 mg/L

[1] AUC (Area Under Curve according to EU Method C.3) means the integral of the biomass. Calculation see under 8.2.

[2] Yield (according to OECD Guideline 201) is defined as the biomass at the end of the exposure period minus the biomass at the start of the exposure period. Calculation see under 8.3

4 PURPOSE AND PRINCIPLE OF THE STUDY

This study was performed in order to evaluate the toxic potential of [redacted] towards *Desmodesmus subspicatus* CHODAT, an unicellular freshwater green alga, which belongs to the order of chlorococcales. This freshwater green alga was chosen as a typical part of phytoplankton. The incubation period of 72 hours corresponds to a subchronic test, as during this period, about five cell divisions take place.

The system response was the reduction of growth in a series of algal cultures (test units) exposed to various concentrations of a test substance. The response was evaluated as a function of the exposure concentration in comparison with the average growth of replicate, unexposed control cultures. For full expression of the system response to toxic effects (optimal sensitivity), the cultures were allowed unrestricted exponential growth under nutrient sufficient conditions and continuous light for a sufficient period of time to measure reduction of the specific growth rate.

Sponsor's intent: registration following REACH.

5 LITERATURE

The study was conducted in accordance with the following guidelines:

- OECD Guideline for the Testing of Chemicals No. 201, adopted 23. Mar. 2006 "Freshwater Alga and Cyanobacteria, Growth Inhibition Test"
- Regulation EC No. 440/2008, Guideline C.3: "Algal inhibition test," adopted 31. May 2008

Corresponding SOP of [redacted]

- [redacted]

Further literature used for the evaluation of the results:

- "Angewandte Statistik für Naturwissenschaftler", Lozán/Kausch, Parey Buchverlag Berlin 1998

6 MATERIALS AND METHODS

6.1 Test Item

6.1.1 Specification

The following information concerning identity and composition of the test item was provided by the sponsor.

Name [redacted]
Batch no.
Appearance
Composition
CAS No.
EINECS-No.
Molecular formula
Molecular weight
Purity
Homogeneity
Vapour pressure
Stability
Solubility
Production date
Expiry date
Storage
Hazard information
R-phrases
S-phrases

6.1.2 Storage

The test item was stored in a closed vessel dark and dry at room temperature.

6.1.3 Preparation

Before each experiment, a saturated solution was prepared. This was done by shaking the nominal load with the appropriate amount of deionised water for 24 hours, followed by membrane filtration. The concentrations to be tested were prepared by dilution of this solution with deionised water. Since the solutions were diluted by algal pre-culture and nutrient medium, 1.25 times the nominal amount was used in preparation of the saturated solution.

6.2 Positive Control

Potassium dichromate $K_2Cr_2O_7$ (CAS No. 7778-50-9) was used as positive control.

6.3 Test System

Selection of the test system was made following the proposal of the guidelines.

6.3.1 Specification

Unicellular freshwater green alga.

Genus, Species	*Desmodesmus subspicatus*
Strain	CHODAT
Family	Chlorophyceae
Order	Chlorococcales

6.3.2 Origin and Culture

The culture of *Desmodesmus subspicatus* was obtained in [redacted] by [redacted] The algae are kept as stock culture on solid agar at 8 °C. From an aliquot of this stock culture the pre-culture was prepared.

6.4 Chemicals

All solutions were sterilised before use.

6.4.1 Composition of the Solutions Algal Toxicity Test:

6.4.1.1 Stock Solution I

NH_4Cl	1500 mg
$MgCl_2*6H_2O$	1200 mg
$CaCl_2*2H_2O$	1800 mg
$MgSO_4*7H_2O$	1500 mg
KH_2PO_4	160 mg
H_2O deionised	ad 1000 mL

6.4.1.2 Stock Solution II

$FeCl_3*6H_2O$	64 mg
$Na_2EDTA*2H_2O$	100 mg
H_2O deionised	ad 1000 mL

The composition of stock solution II is taken from OECD Guideline 201. As in EU-Method C.3, a different composition (80 mg $FeCl_3*6H_2O$/L) is stated, this is stated as a deviation from the EU Method.

6.4.1.3 Stock Solution III

H_3BO_3	185 mg
$MnCl_2*4H_2O$	415 mg
$ZnCl_2$	3 mg
$CoCl_2*6H_2O$	1.5 mg
$CuCl_2*2H_2O$	0.01 mg
$Na_2MoO_4*2H_2O$	7 mg
H_2O deionised	ad 1000 mL

6.4.1.4 Stock Solution IV

$NaHCO_3$	50 g
H_2O deionised	ad 1000 mL

6.4.1.5 Pre-culture-medium

Stock solution I	10.0 mL
Stock solution II	1.0 mL
Stock solution III	1.0 mL
Stock solution IV	1.0 mL
H_2O deionised	ad 1000 mL

6.4.1.6 Nutrient-medium (10-fold concentrated)

Stock solution I	100 mL
Stock solution II	10 mL
Stock solution III	10 mL
Stock solution IV	10 mL
H_2O deionised	ad 1000 mL

6.4.2 Composition of Chemicals for Analytical Determinations

6.4.2.1 Hydrochloric Acid

HCl conc., p.A., 37%

6.4.2.2 Potassium Chloride Solution

KCl, p.A., 10 % solution in H_2O demin.

6.5 Instruments and Devices

The following instruments and devices were used for this study:

- Analytical Scales Mettler XS 205 DU [redacted] No. 2
- Luxmeter LT lutron LX-101
- Glass thermometer: [redacted]
- Lighting incubator JPL
- Orbital shakers GFL 3019 ([redacted] No. 6) and GFL 3017 [redacted] No. 10)
- Glass measuring cylinders and glass measuring flasks
- Automatic pipettes with one-way tips, [redacted] No.s 14, 21, 23, 25, 26, 30, 31
- pH-meter 340i wtw
- Photometer Specord 205 Analytik Jena
- Microscope Axiolab
- Magnetic stirrer
- AAS contrAA300 Analytik Jena AG

Usage and, if applicable, calibration of all instruments following the corresponding SOP in the current edition.

6.6 Analytical Method

The content of the test item [redacted] in the test solutions was determined by measurement of [redacted] as a component of the test item using AAS. AAS measurements were performed with the flame technique (Air/N_2O) at [redacted] nm for [redacted]

Before the start of the algal toxicity test, accuracy and stability of the test item in test medium was tested.

6.6.1 Sample Preparation

To all samples, 1 % KCl solution (10% in water) and 1% HCl conc. were added. On each day of the measurements, calibrations were performed in the range 0.01 – 2 mg/L [redacted]

The lowest value of calibration (0.01 mg/L) was defined as limit of quantification (LOQ) and limit of detection (LOD).

6.6.2 Stability of Solutions

The recovery rate for the test item at a concentration of 100.9 mg/L (nominal) after 72 hours was determined as 68.6 %. Thus the test item must be stated as not stable under test conditions. The details are given in the following table.

Table 6.6-a Recovery Rates

Time in Days	Measured Conc [redacted] n mg/L	Mean	Stand. Dev.	RSD
0	0.1226			
0	0.1033			
0	0.1239	0.1166	0.01153646	9.90%
3	0.0787			
3	0.0719			
3	0.0892	0.0799	0.0087157	10.90%
Stability	**68.60%**			

6.6.3 Accuracy

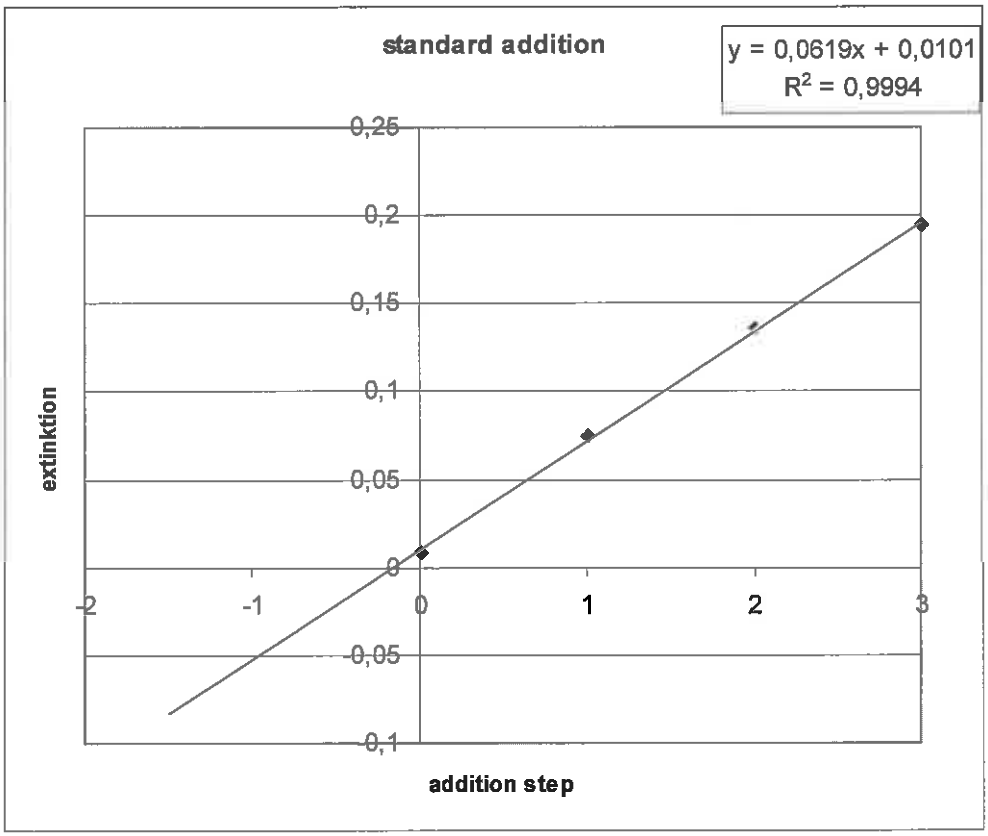
The accuracy in algal medium was determined via standard addition. To the solution from the stability test at t = 3 days, 1% HCl conc. and 1% KCl 10% were added. Then, the solution was diluted 1:100. Different amounts (0; 150 µl; 300 µl; 450 µl) of a stock solution (c [redacted] = 10 mg /L) were added to 10 ml diluted test solution.

Accuracy was given, the values showed a linear correlation with r > 0.99.

Table 6.6-b Accuracy in Medium

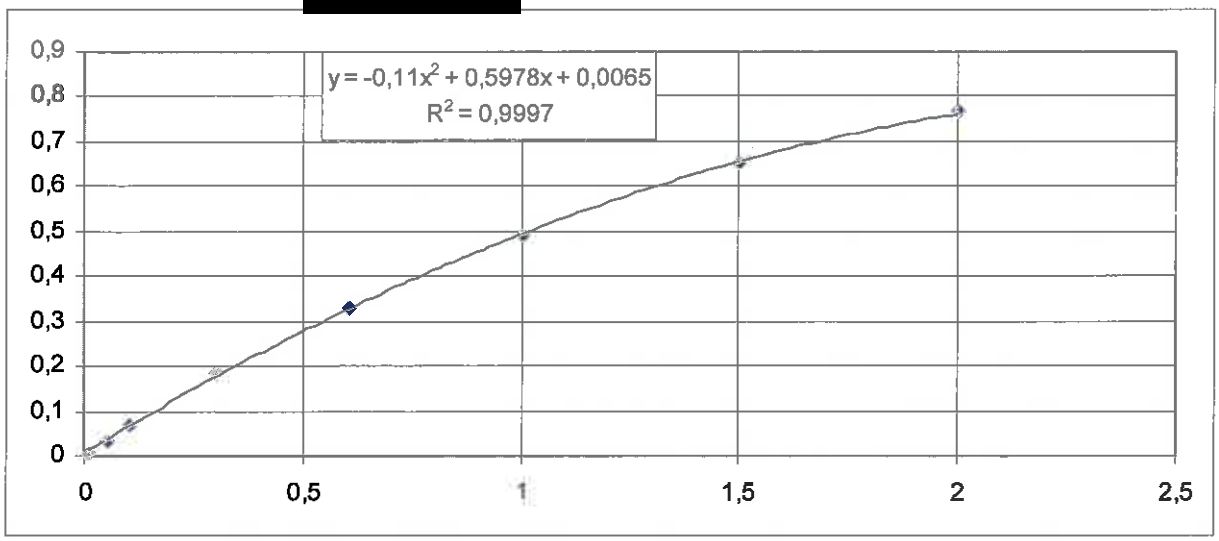
Addition	Absorption Values	Parameter	Value
0	0.00823	Slope	0.061876
1	0.07399	Intercept	0.010051
2	0.13514	r	0.99969056
3	0.1941	r^2	0.99938122

Graph 6.6-I Accuracy

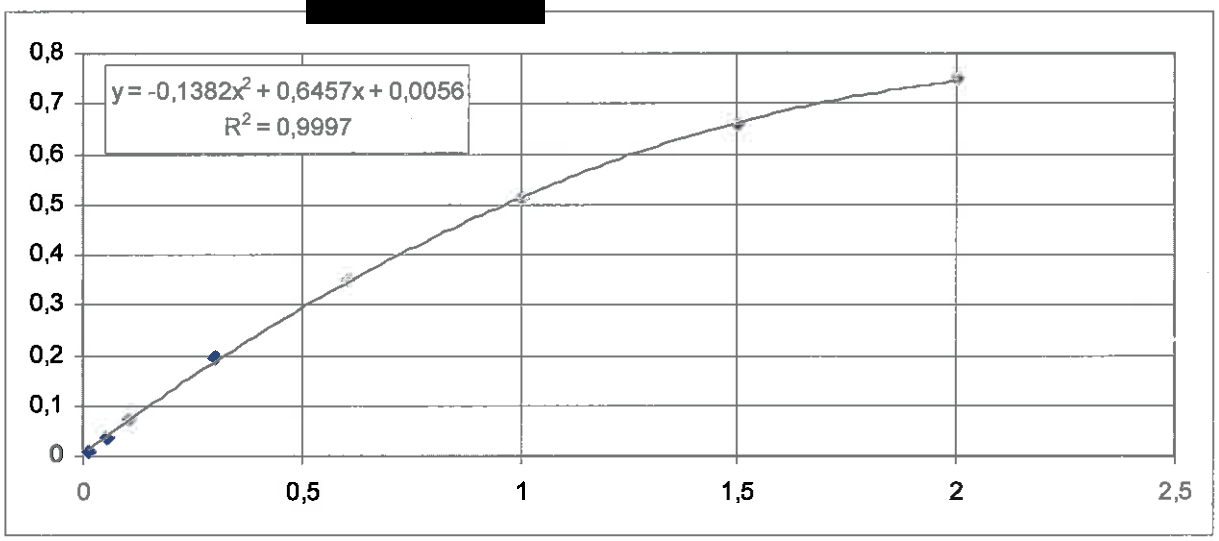


6.6.4 Calibration Curves

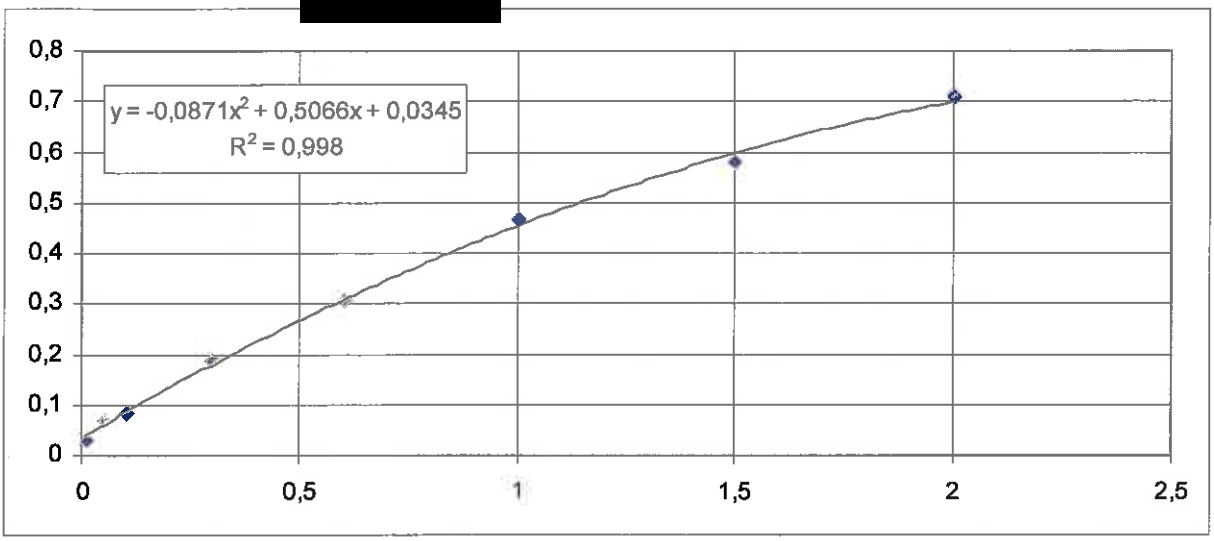
6.6.4.1 Calibration of [redacted]



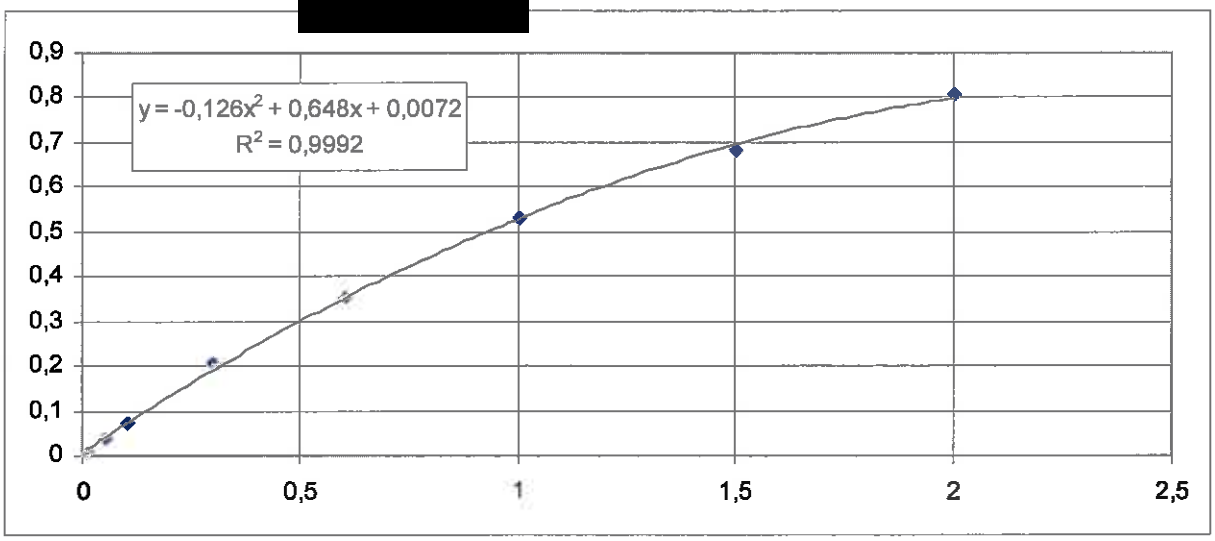
6.6.4.2 Calibration of [redacted]



6.6.4.3 Calibration of [redacted]



6.6.4.4 Calibration of [redacted]



For each run, the settings and parameters were documented.
All AAS data are archived following GLP regulations.

7 CONDUCT OF THE STUDY

7.1 Alga

Four days before the start of the test, an aliquot of the stock culture containing a few cells was brought into pre-culture medium and incubated for 72 hours. The resulting culture is growing exponentially.

Before usage, the culture was checked on the absence of cell aggregates. After the adjustment to a cell concentration of about $6*10^4$/mL through photometric measurement and addition of algal medium, the culture was usable for the test. The adjusted pre-culture was mixed with the same amount of 10-fold-nutrient solution. This mixture was the test culture.

7.2 Preparations

On the day before the start of the test, a sufficient number of vessels were provided. Two hours before the start, the light incubator was turned on in order to warm up.

7.3 Main Study

Two experiments were performed. In the first experiment the validity criteria were not met. Therefore, this experiment was considered as invalid and was repeated. The results of the first experiment aren't reported.

7.3.1 Performance of the Study

For each treatment, 240 mL of the respective test solution (1.25-fold concentrated) was prepared and mixed with 60 mL of the test culture. In this mixture, the pH-value was measured. A sample for the analytical determination was taken.

The test vessels were filled with 40 ± 5 mL of the mixture and were incubated open for 72 hours, shaken on an orbital shaker. Before the start of incubation and every 24 hours, the cell number was determined and the values of the absorption were recorded. After the test, the pH value in treatments and control was measured once more.

At the end of the test, the treatments were observed microscopically in order to assess the appearance of the alga and detect abnormalities (e.g. caused by the exposure to the test item).

The content of [redacted] in the test vessels was measured at the start, every 24 hours and at the end of the test.

7.3.2 Experimental Conditions

Date:	[redacted]
Treatments tested:	1.0 / 3.2 / 10 / 32 / 100 mg/L
Number of replicates:	seven replicates for the control four replicates for each treatment (one replicate control and each treatment only for analytical determination)
Vessels:	glass flasks 50 mL
Duration:	72 hours
Temperature:	27 °C
Lighting:	4600 – 4700 Lux
Control:	deionised water with nutrient medium and alga
Treatments:	test solution with nutrient medium and alga

7.4 Reference Study

7.4.1 Performance of the Study

For each treatment, 40 mL of the respective test solution (1.25-fold concentrated) was prepared and mixed with 10 mL of the test culture. In this mixture, the pH-value was measured.

The test vessels were filled with 5 mL of the mixture and were incubated open for 72 hours stirred with magnetic stirrers. Before the start of incubation and every 24 hours, the cell number was determined and the values of the absorption of the cuvettes were recorded. After the test, the pH value in treatments and control was measured once more.

At the end of the test, the treatments were observed microscopically in order to assess the appearance of the alga and detect abnormalities (e.g. caused by the exposure to the positive control).

7.4.2 Preparation of the Test Solutions

A stock solution containing 250 mg/L positive control in deionised water was prepared.

7.4.3 Study Parameters

Date:	[redacted]
Treatments tested:	0.04 / 0.063 / 0.1 / 0.16 / 0.25 / 0.4 / 0.63 / 1.0 / 1.6 mg/L
Number of replicates:	six replicates for the control three replicates for each treatment
Vessels:	round cuvettes d = 10 mm
Duration:	72 hours
Temperature:	25 - 27 °C
Lighting:	8500 – 8600 Lux
Control:	deionised water with nutrient medium and alga
Treatments:	test solution with nutrient medium and alga

8 CALCULATIONS

The data were evaluated after the test with Excel®.

8.1 Growth Rate

The specific growth rate μ was calculated using the following equation:

$$\mu = \frac{\ln(N_n) - \ln(N_0)}{t_n}$$

with:

μ	growth rate;
t_n	time of the last measurement in days
N_0	nominal number of cells at time t_0
N_n	nominal number of cells at time t_n

8.2 Area under the Growth Curves – Bio Mass Integral (AUC)

Area AUC below the growth curve (AUC) was calculated using the following equation:

$$AUC = \frac{N_1 - N_0}{2} * t_1 + \frac{N_1 + N_2 - 2 * N_0}{2} * (t_2 - t_1) + \ldots + \frac{N_{n-1} + N_n - 2 * N_0}{2} * (t_n - t_{n-1})$$

with:

t_1	time of first measurement
t_n	time of n^{th} measurement
N_0	nominal number of cells at time t_0
N_1	nominal number of cells at time t_1
N_n	nominal number of cells at time t_n

8.3 Yield

The yield was calculated using the following equation:

$$Yield = N_{72} - N_0$$

with:

N_0	number of cells at time t = 0h
N_{72}	number of cells at time t = 72h

9 FINDINGS

9.1 Main Study

9.1.1 Cell Numbers

The cell numbers were determined by photometric measurement of optical density (absorption values are given in the annex, page 40). Cell numbers of individual replicates are given in the annex, also. The means and standard deviations of the cell numbers of the control and the treatments are presented in the following table:

Table 9.1-a Cell Number/mL Main Study

Nominal Concentration in mg/L	Parameter	Cell Number/mL			
		0 h	24 h	48 h	72 h
0	Mean	15148	61854	204498	751088
0	SD	0	5702	34209	139033
1.0	Mean	15148	70691	302960	1030064
1.0	SD	0	17491	20039	198664
3.2	Mean	15148	53018	229745	994719
3.2	SD	0	7574	41714	64416
10	Mean	15148	50493	191875	429193
10	SD	0	8746	15767	44380
32	Mean	15148	37870	68166	83314
32	SD	0	0	7574	7574
100	Mean	15148	53018	60592	60592
100	SD	0	0	7574	7574

SD = Standard Deviation

9.1.2 Temperature, Light Intensity, pH

The pH values in the control ranged from 7.7 to 8.6. Light intensity was in a range of 4600 – 4700 lux. Temperature was 27.0 °C. The details are given in the following table:

Table 9.1-b Temperature, Light Intensity, pH Main Study

		0 h	24 h	48 h	72 h
Temperature [°C]		27	27	27	27
Light intensity [lux]		4600	4600	4600	4700
pH	Conc. in mg/L		-	-	
	0	7.7	-	-	8.6
	1.0	7.7	-	-	10.1
	3.2	7.8	-	-	9.5
	10	7.7	-	-	8.2
	32	7.7	-	-	7.8
	100	7.6	-	-	7.8

9.1.3 Microscopical Observation

In the following table the appearance of the alga at the end of the test were stated:

Table 9.1-c Microscopical Observation Main Study

Nom. Conc. in mg/L	Observation
0	Normal growth
1.0	Normal growth
3.2	Normal growth
10	Less cells
32	No cells
100	No cells

9.1.4 Analytical Determination

The content of [redacted] part of the test item) in the test solution was analytically determined using AAS. As the test item wasn't stable under test conditions, analytical measurement were performed every day of the test. In treatments 1 mg/L and 3.2 mg/L (nominal concentrations), [redacted] was only detectable at the beginning and after 24 hours .For these treatments, only these two measured concentrations were used for calculation of the geometric mean and evaluation of the results. The low measured [redacted] content in the control at the beginning and in treatment 3.2 mg/l after 72 hours might be caused by contaminated vessels. Because of the poor solubility in test medium, the correlation between nominal and measured concentration was poor.

Therefore, the geometric mean of the measured concentrations was used for the determination of the results.

The details are given in the following table:

Table 9.1-d Measured Concentrations in mg/L and corresponding Recovery Rates in %

Nom. Conc. in mg/L	Conc. [redacted] mg/L 0h	Conc. mg/L 24h	Conc. mg/L 48h	Conc. mg/L 72h	Conc. Test Item mg/L 0h	Conc. Test Item mg/L 24h	Conc. Test Item mg/L 48h	Conc. Test Item mg/L 72h	geom. Mean mg/L	% of Nom. Conc.
0	0.045	n. d.	n. d.	n. d.	--	--	--	--	--	--
1	0.040	0.030	n. d.	n. d.	0.301	0.225	--	--	0.26	26
3.2	0.087	0.097	n. d.	*0.031	0.659	0.731	--	*0.231	0.69	22
10	0.435	0.327	0.218	0.203	3.283	2.470	1.646	1.529	2.13	21
32	2.052	2.076	1.051	1.590	15.491	15.665	7.929	11.998	12.33	39
100	5.680	5.090	3.055	3.318	42.868	38.413	23.054	25.040	31.22	31

n. d. = not detectable

*** not used for evaluation of the results**

9.1.5 Area under the Curve, Growth Rate, Yield

From the cell numbers, the Growth Rate µ, the Area under the Curve AUC, and the Yield were calculated. The means and standard deviations at the end of the test are given in the following table:

Table 9.1-e Growth Rate µ, Area under the Curve AUC, Yield Main Study

Nom. Concentration in mg/L	Meas. Concentration in mg/L		Growth Rate [day^{-1}]	AUC [Cell Concentration/mL*day]	Yield [Cell Concentration/mL]
0	0	Mean	1.30	604027	735940
		SD	0.07	99267	139033
1.0	0.26	Mean	1.40	850813	1014916
		SD	0.07	107202	198664
3.2	0.69	Mean	1.39	742252	979571
		SD	0.02	72548	64416
10	2.13	Mean	1.11	419095	414045
		SD	0.04	44218	44380
32	12.33	Mean	0.57	109823	68166
		SD	0.03	10019	7574
100	31.22	Mean	0.46	106036	45444
		SD	0.04	11361	7574

SD = Standard Deviation

9.1.6 Inhibition

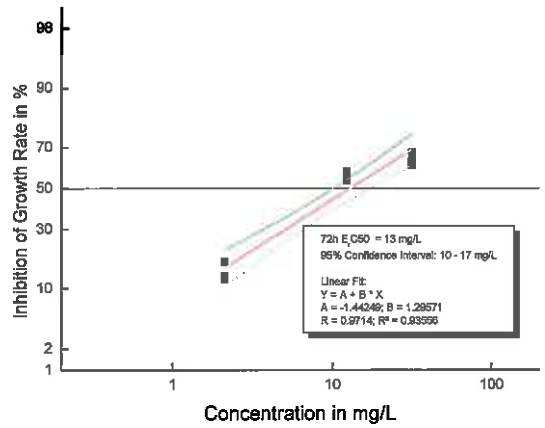
The following mean inhibition values were calculated for the Growth Rate µ, the Area under the Curve AUC, and the Yield. Individual inhibition values are given in the annex.

Table 9.1-f Inhibition Values Main Study

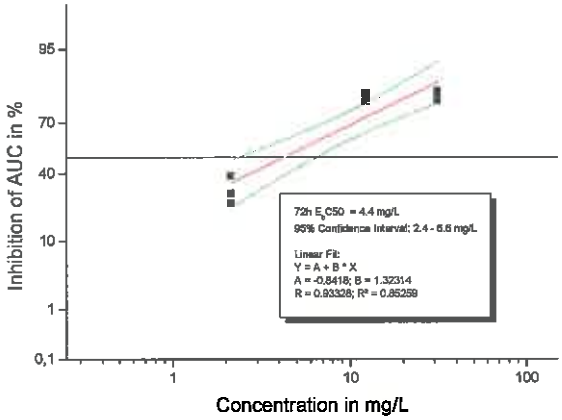
Nom. Concentration in mg/L	Meas. Concentration in mg/L	% Inhibition		
		Growth Rate µ	Area under the Curve AUC	Yield
0	0	0!	0!	0!
1.0	0.26	-8.18	-40.86	-37.91
3.2	0.69	-7.59	-22.88	-33.10
10	2.13	14.09	30.62	43.74
32	12.33	56.23	81.82	90.74
100	31.22	64.48	82.45	93.83

9.1.7 Graphs Inhibition Main Study

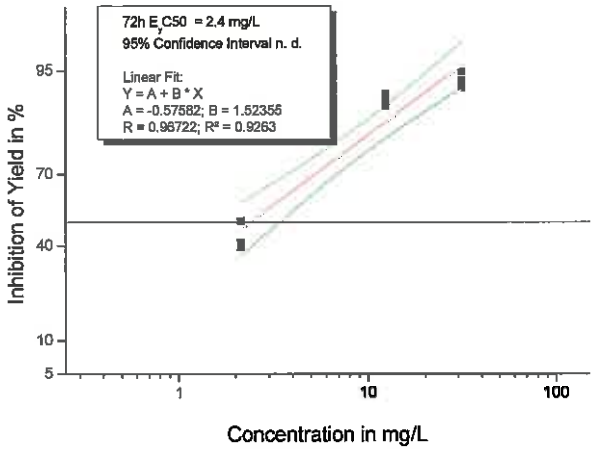
9.1.7.1 Inhibition Growth Rate



9.1.7.2 Inhibition Area under the Curve (Biomass Integral)



9.1.7.3 Inhibition Yield



9.2 Reference Study

9.2.1 Cell Numbers

The cell numbers were determined by photometric measurement of optical density (absorption values are given in the annex, page 47). Cell numbers of individual replicates are given in the annex, also. The means and standard deviations of the cell numbers of the control and the treatments are presented in the following table:

Table 9.2-a Cell Number/mL Reference Study

Nominal Concentration in mg/L	Parameter	Cell Number/mL			
		0 h	24 h	48 h	72 h
0	Mean	15148	65641	273926	961898
0	SD	0	7822	31925	148342
0.046	Mean	15148	58067	244893	850813
0.046	SD	0	4373	11569	21864
0.063	Mean	15148	58067	244893	828091
0.063	SD	0	4373	15767	19061
0.1	Mean	15148	68166	239843	800319
0.1	SD	0	7574	15767	43068
0.16	Mean	15148	65641	257516	764974
0.16	SD	0	4373	20039	118310
0.25	Mean	15148	60592	209547	636216
0.25	SD	0	7574	4373	22722
0.4	Mean	15148	68166	181776	436767
0.4	SD	0	15148	7574	8746
0.63	Mean	15148	50493	103511	199449
0.63	SD	0	8746	4373	4373
1	Mean	15148	42919	65641	85839
1	SD	0	4373	4373	11569
1.6	Mean	15148	35345	40395	55543
1.6	SD	0	4373	8746	8746

SD = Standard Deviation

9.2.2 Temperature, Light Intensity, pH

The pH values in the control ranged from 7.6 to 7.7. The light intensity was 8500 – 8600 lux. Temperature was in a range of 25 – 27 °C. The details are given in the following table:

Table 9.2-b Temperature, Light Intensity, pH Reference Study

		0 h	24 h	48 h	72 h
Temperature [°C]		25	26	27	27
Light intensity [lux]		8500	8600	8600	8600
pH	**Conc. in mg/L**		-	-	
	0	7.7	-	-	7.6
	0.04	8.5	-	-	7.5
	0.063	8.5	-	-	7.5
	0.1	8.5	-	-	7.5
	0.16	8.5	-	-	7.5
	0.25	8.5	-	-	7.5
	0.4	8.5	-	-	7.6
	0.63	8.5	-	-	7.6
	1.0	8.5	-	-	7.5
	1.6	8.5	-	-	7.7

9.2.3 Microscopical Observation
In the following table the appearance of the alga at the end of the test were stated:

Table 9.2-c Microscopical Observation Reference Study

Nom. Conc. in mg/L	Observation
0	Normal growth
0.04	Normal growth
0.063	Normal growth
0.1	Normal growth
0.16	Normal growth
0.25	Normal growth
0.4	Less cells
0.63	Less cells
1.0	No cells
1.6	No cells

9.2.4 Area under the Curve, Growth Rate, Yield
From the cell numbers, the Growth Rate μ, the Area under the Curve AUC, and the Yield were calculated. The means and standard deviations at the end of the test are given in the following table:

Table 9.2-d Growth Rate μ, Area under the Curve AUC, Yield Reference Study

Nominal Concentration in mg/L		Growth Rate [day^{-1}]	AUC [Cell Concentration/mL*day]	Yield [Cell Concentration/mL]
0	Mean	1.38	782647	946750
	SD	0.05	101973	148342
0.04	Mean	1.34	690496	835665
	SD	0.01	25215	21864
0.063	Mean	1.33	679135	812943
	SD	0.01	24640	19061
0.1	Mean	1.32	670299	785171
	SD	0.02	44648	43068
0.16	Mean	1.30	667774	749826
	SD	0.05	80986	118310
0.25	Mean	1.25	550377	621068
	SD	0.01	20857	22722
0.4	Mean	1.12	430456	421619
	SD	0.01	4373	8746
0.63	Mean	0.86	215859	184301
	SD	0.01	13654	4373
1.0	Mean	0.58	113610	70691
	SD	0.04	3787	11569
1.6	Mean	0.43	65641	40395
	SD	0.06	17491	8746

9.2.5 Inhibition

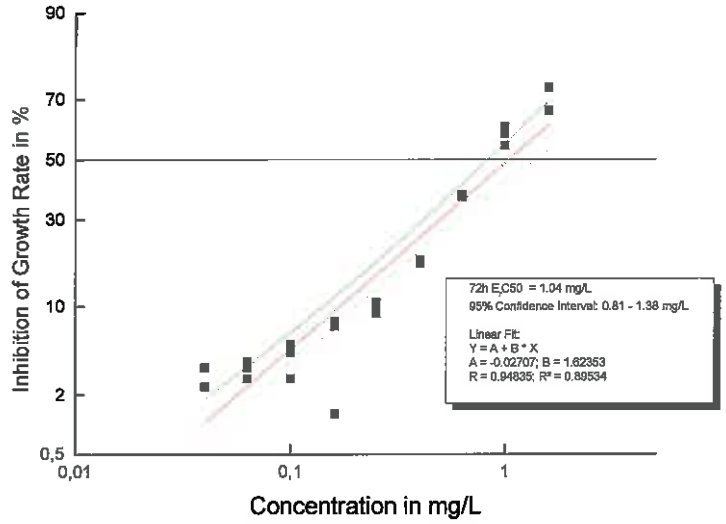
The following mean inhibition values were calculated for the Growth Rate µ, the Area under the Curve AUC, and the Yield. Individual inhibition values are given in the annex.

Table 9.2-e Inhibition Values Reference Study

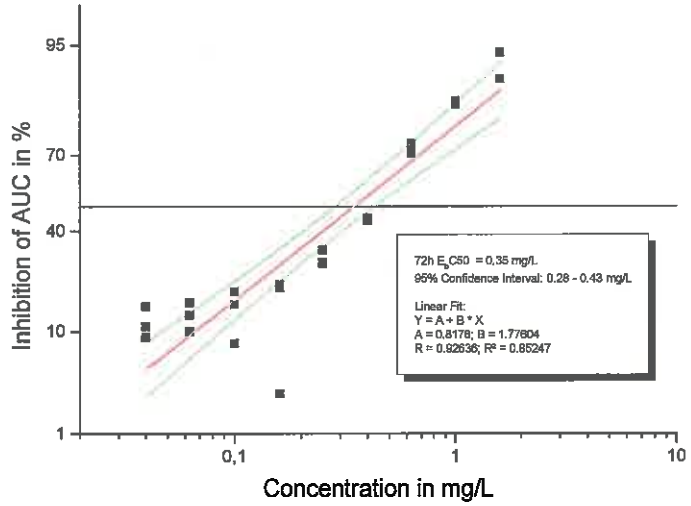
Nominal Concentration in mg/L	% Inhibition		
	Growth Rate µ	Area under the Curve AUC	Yield
0	0!	0!	0!
0.04	2.71	11.77	11.73
0.063	3.36	13.23	14.13
0.1	4.21	14.35	17.07
0.16	5.46	14.68	20.80
0.25	9.74	29.68	34.40
0.4	18.81	45.00	55.47
0.63	37.75	72.42	80.53
1.0	58.25	85.48	92.53
1.6	68.83	91.61	95.73

9.2.6 Graphs Inhibition Reference Study

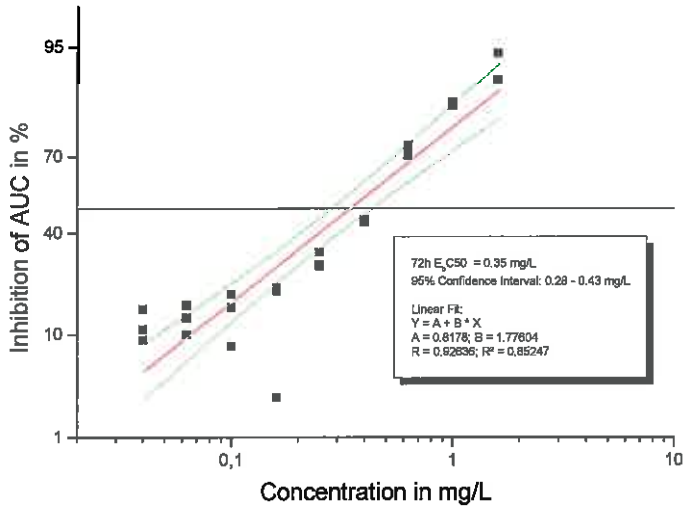
9.2.6.1 Inhibition Growth Rate



9.2.6.2 Inhibition Area under the Curve (Biomass Integral)



9.2.6.3 Inhibition Yield



10 STATISTICS AND RESULTS

10.1 Significance

For the treatments with the nominal concentrations 3.2 and 10 mg/L (measured concentrations 0.69 and 2.13 mg/L), it was tested whether the differences between treatment and control were significant. The values for the area under the curve, Yield and the growth rate were used for the assessment of observed effect levels.

In order to select a suitable test for significance, it was checked whether equality of variance was given.

Equality of variance is tested using the following equation:

$$F = \frac{s_1^2}{s_2^2}$$

with s_1 being the greater variance and s_2 the smaller one.

The calculated value F is compared with the F-test table (level of significance 95%). If the calculated value is smaller than the tabular value, equality of variance is given.

Table 10.1-a Mean, Variance, Standard Deviation and F-Value

Nominal Conc.	**Control**			**3.2 mg/L (0.69 mg/L measured conc.)**		
Endpoint	**µ**	**AUC**	**Yield**	**µ**	**AUC**	**Yield**
Mean	1.30	604027	735940	1.39	742252	979571
Standard Deviation	0.07	99267	139033	0.02	72548	64416
Variance	0.00	9.854E+09	1.933E+10	0.0005	5.263E+09	4.149E+09
F-Value (Equality of Variance with Control)				9.21	1.87	4.66
F-Value taken from the table (5 %)				19.30	19.30	19.30
Equality of Variance given?				yes	yes	yes
Nominal Conc.				**10 mg/L (2.13 mg/L measured conc.)**		
Endpoint				**µ**	**AUC**	**Yield**
Mean				1.11	419095	414045
Standard Deviation				0.04	44218	44380
Variance				0.0013	1.955E+09	1.970E+09
F-Value (Equality of Variance with Control)				3.40	5.04	9.81
F-Value taken from the table (5%)				19.30	19.30	19.30
Equality of Variance given?				yes	yes	yes

In this table, E+09 represents multiplication with 10^9.

If equality of variance is given, the t-test is used; else, the WEIR test is used.

Equation for the calculation of t:

$$t = \frac{\left|\bar{x}1 - \bar{x}2\right|}{(\sqrt{\sum xx1 + \sum xx2})/(n1 + n2 - 2)} \sqrt{\frac{n1 * n2}{n1 + n2}}$$

with:

x1	value control
x2	value treatment
$\bar{X}_1$	mean value control
$\bar{X}_2$	mean value treatment
$\sum xx1$	$\sum x1^2-(\sum x1)^2/n$
$\sum xx2$	$\sum x2^2-(\sum x2)^2/n$
n1	number of replicates control (6)
n2	number of replicates treatment (3)

With the t-Test. it was checked whether the differences are significant. Significance is given if the calculated t-value is bigger than the limit of significance (t-value taken from the table with grade of freedom: n1 + n2 – 2, level of significance 95 %).

Table 10.1-b Significance

Nominal Conc.	3.2 mg/L (0.69 mg/L measured conc.)			10 mg/L (2.13 mg/L measured conc.)		
Endpoint	**µ**	**AUC**	**Yield**	**µ**	**AUC**	**Yield**
calculated t-value	2.46	2.12	2.81	4.42	3.00	3.80
tabulated t-value	2.36	2.36	2.36	2.36	2.36	2.36
Significance given?	no	no	no	yes	yes	yes

10.1.1 Determination of the NOEC

For the endpoint AUC, the differences between the treatment 3.2 mg/L nominal concentration (0.69 mg/L measured concentration) and the control can be considered as not significant (level of significance: 97.5 %) as the calculated t-value was smaller than the limit of significance.

For the endpoints µ and Yield, the calculated t-values were bigger than the limit of significance. Algal growth in treatment 3.2 mg/L nominal concentration (0.69 mg/L measured concentration) was significantly higher than in the control. Therefore, the concentration 0.69 mg/L can be stated as NOEC respectively as NOAEC (no observed adverse effect concentration) for these endpoints.

10.1.2 Determination of the LOEC

For the endpoints µ, AUC and Yield, the differences between the treatment 10 mg/L nominal concentration (2.13 mg/L measured concentration) and the control must be considered as significant (level of significance: 97.5 %), the concentration 2.1 mg/L can be stated as LOEC for these endpoints.

10.2 Biological Results of the Test Item

The estimation of the EC50s of the test item was accomplished using the software Origin™. The calculated values for r resp. r^2 are given in the graph.

The data were evaluated using linear fit on a probability-logarithmic scale.

Equation: $Prob(y) = A + B * \log (x)$

with

y Inhibition in %

x Concentration in mg/L

The following values were determined:

Table 10.2-a Biological Results of the Test Item

Parameter	Geometric Mean of measured Conc.	95%-confidence-interval
NOEC (Growth Rate) 72 h	0.69 mg/L	not determinable
NOEC (AUC) 72 h	0.69 mg/L	not determinable
NOEC (Yield) 72 h	0.69 mg/L	not determinable
LOEC (Growth Rate) 72 h	2.1 mg/L	not determinable
LOEC (AUC) 72 h	2.1 mg/L	not determinable
LOEC (Yield) 72 h	2.1 mg/L	not determinable
72h E_rC50	13 mg/L	10 – 17 mg/L
72h E_bC50	4.4 mg/L	2.4 – 6.5 mg/L
72h E_yC50	2.4 mg/L	not determinable
72h-E_rC100	> 31 mg/L	not determinable
72h-E_bC100	> 31 mg/L	not determinable
72h-E_yC100	> 31 mg/L	not determinable

10.3 Biological Results of the Positive Control

The estimation of the EC50s of the positive control was accomplished using the software Origin™. The calculated values for r resp. r^2 are given in the graph.

The data were evaluated using linear fit on a probability-logarithmic scale.

Equation: $Prob(Y) = A + B * \log (X)$

with

y Inhibition in %

x Concentration in mg/L

The following values were determined:

Table 10.3-a Biological Results of the Positive Control

Parameter	Value	95%-confidence-interval	Range of interlaboratory test (EU C.3)
NOEC 72 h	0.16 mg/L	not determinable	none stated
LOEC 72 h	0.25 mg/L	not determinable	none stated
72h E_rC50	1.0 mg/L	0.81 – 1.4 mg/L	0.60 – 1.0 mg/L
72h E_bC50	0.35 mg/L	0.28 – 0.43 mg/L	0.20 – 0.75 mg/L
72h E_yC50	0.26 mg/L	0.22 – 0.31 mg/L	none stated

11 VALIDITY

11.1 Growth Rate

11.1.1 Increase Factor

The cell concentration in the control should increase by a factor of at least 16 within 72h. The following increase factors were reached:

Table 11.1-a Increase Factor of the Control

Study	Increase Factor	Assessment
Reference Study	64	ok
Main Study	50	ok

11.1.2 Daily Growth Rates

The daily growth rates of the controls were calculated. Means, standard deviations and coefficients of variation were determined. Values and assessment can be found in the following tables.

11.1.2.1 Main Study

Table 11.1-b Daily Growth Rates of the Controls

Growth rates	days 0 – 1	days 1 – 2	days 2 – 3	CV of sectional daily growth rates	days 0 – 3
Replicate 1	1.386	0.965	1.190	18%	1.18
Replicate 2	1.386	1.099	1.376	13%	1.29
Replicate 3	1.253	1.232	1.476	10%	1.32
Replicate 4	1.386	1.355	1.181	8%	1.31
Replicate 5	1.504	1.237	1.402	10%	1.38
Replicate 6	1.504	1.237	1.161	14%	1.30
Mean	1.403	1.187	1.298	**12%**	1.296
Standard deviation	0.094	0.136	0.136		0.065
CV	7%	11%	10%		**5%**

CV = Coefficient of Variation

For the main study, the calculated parameters are assessed as follows:

Table 11.1-c Assessment

Parameter	Criteria	Value	Assessment
Increase factor of Cell Concentration	factor 16 in 72 h	50	valid
Mean coefficient of variation of daily growth rates	max. 35%	12 %	valid
Coefficient of variation of average growth rate during the whole test period	max. 7%	5 %	valid
Exponential growth during the whole test period given? (graphical assessment)			yes

11.1.2.2 Reference Study

Table 11.1-d Daily Growth Rates of the Controls

Growth rates	days 0 – 1	days 1 – 2	days 2 – 3	CV of sectional daily growth rates	days 0 – 3
Replicate 1	1.386	1.558	1.282	10%	1.41
Replicate 2	1.386	1.634	1.221	15%	1.41
Replicate 3	1.386	1.584	1.234	13%	1.40
Replicate 4	1.386	1.355	1.229	6%	1.32
Replicate 5	1.609	1.131	1.161	21%	1.30
Replicate 6	1.609	1.308	1.380	11%	1.43
Mean	1.461	1.428	1.251	**12%**	1.380
Standard deviation	0.115	0.196	0.074		0.054
CV	8%	14%	6%		**4%**

CV = Coefficient of Variation

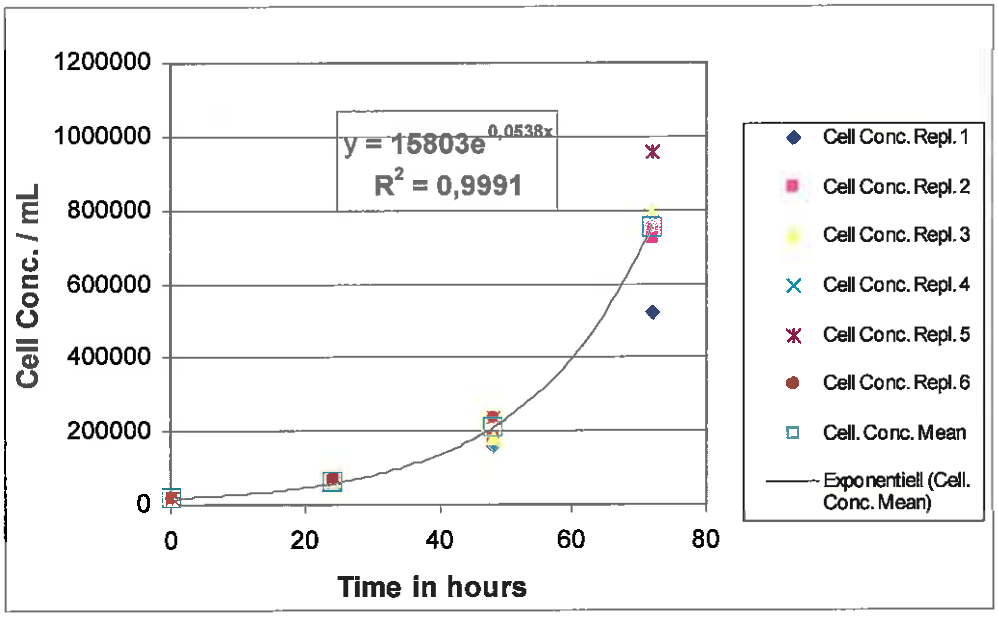
For the reference study, the calculated parameters are assessed as follows:

Table 11.1-e Assessment

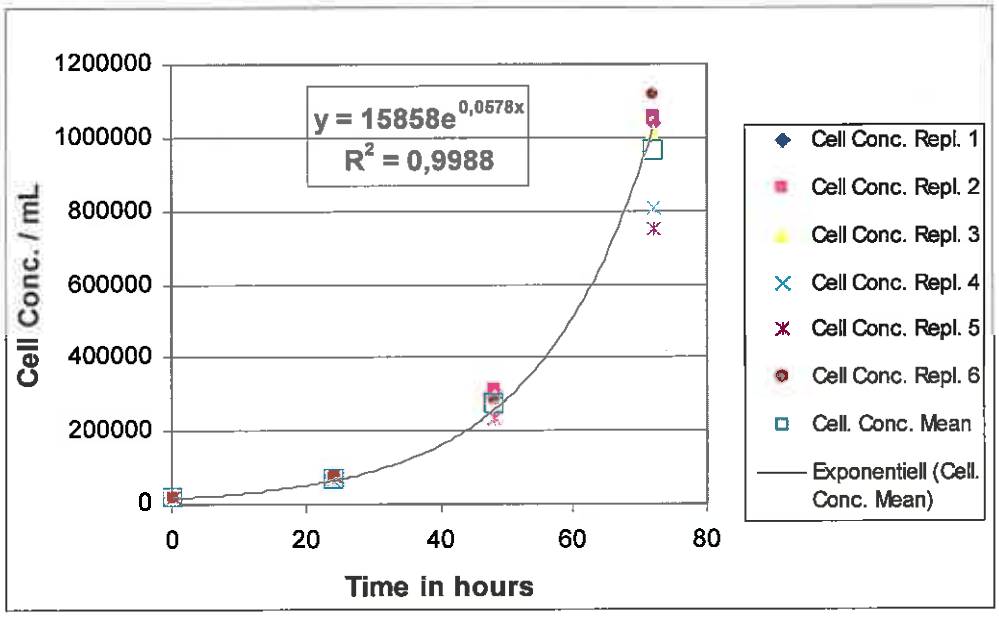
Parameter	Criteria	Value	Assessment
Increase factor of Cell Concentration	factor 16 in 72 h	64	valid
Mean coefficient of variation of daily growth rates	max. 35%	12%	valid
Coefficient of variation of average growth rate during the whole test period	max. 7%	4%	valid
Exponential growth during the whole test period given? (graphical assessment)			yes

11.2 Growth Curves

11.2.1 Main Study



11.2.2 Reference Study



11.3 Change of pH

The pH of the control shouldn't change by more than 1.5 units. The change was 0.1 units in the reference study and 0.9 units in the main study.

12 Discussion

Two experiments were performed. In the first experiment, the validity criteria were not met. Therefore, this experiment was considered invalid and was repeated. The data of the invalid experiment are not reported but will be kept with the raw data.

In the second experiment, all validity criteria were met.

The EC50s of potassium dichromate were determined in a current reference test. For the estimation of the EC50s of the positive control, the fits showed sufficient statistical correspondence of the data with the dose-response-equation. The values were within the normal range of the laboratory as well as in correspondence with the values from the international ring test which are stated in EU Method C.3.

For the estimation of the EC50s of the test item, the fits showed sufficient statistical correspondence of the data with the dose-response-equation.

As the test item is [redacted] in water, a saturated solution was prepared. This was done by shaking the nominal load with the appropriate amount of deionised water for 24 hours, followed by membrane filtration. The concentrations to be tested were prepared by diluting of this solution with deionised water.

The content of [redacted] (part of the test item) in the test solution was analytically determined using AAS. As the test item [redacted] analytical measurements were performed every day of the test.

In the lowest concentrated treatments 1 mg/L and 3.2 mg/L (nominal concentrations), [redacted] was detectable only at the beginning and after 24 hours.

In treatment 3.2 mg/L after 72 hours and in the control at the beginning of the test, [redacted] was detectable in a range marginally above LOD. This low concentration might be caused by contaminated vessel and can be stated as uncritical for the outcome of the study.

Because of poor solubility in test medium, the correlation between nominal and measured concentration was poor (21 %- 39 %). Therefore, the determination of the results was based on the geometric means of the measured concentrations.

No observations were made which might cause doubts concerning the validity of the study outcome.

13 DEVIATIONS

13.1 Deviations from the Study Plan

The following deviation from the study plan was documented:

- The temperature in the reference study was higher than stated in the guideline. Because the controls showed a normal exponential growth, this is stated as uncritical.

The deviation was signed and assessed by the study director on [redacted]

- The temperature in the main study was higher than stated in the guideline. Because the controls showed a normal exponential growth, this is stated as uncritical.

The deviation was signed and assessed by the study director on [redacted]

- In the main study, mistakenly, only 300 ml test solution was prepared instead of 500 ml. This can be stated as uncritical for the outcome of the study as 300 mL were sufficient.

The deviation was signed and assessed by the study director on [redacted]

13.2 Deviations from the Guideline

See above (temperature).

In OECD Guideline 201 and EU Method C.3, different compositions of stock solution II are stated. The study was conducted in accordance with OECD Guideline 201, therefore, the composition of stock solution II must be stated as deviation from EU Method C.3.

14 RECORDING

One original of study plan and final report, respectively, all raw data of the study and all documents mentioned or referred to in study plan or final report will be kept in the GLP Document Archive of the test facility for fifteen years. After that, the sponsor's instructions will be applied (shipment of documentation to sponsor). A retain sample of the test item will be kept in the GLP Substance Archive for fifteen years; then, the retain sample will be discarded.

Number of originals which will be sent to the sponsor: 1

15 ANNEX 1: COPY OF GLP-CERTIFICATE

RheinlandPfalz
LANDESAMT FÜR UMWELT, WASSERWIRTSCHAFT UND GEWERBEAUFSICHT

GUTE LABORPRAXIS – GOOD LABORATORY PRACTICE

GLP-BESCHEINIGUNG
STATEMENT OF GLP COMPLIANCE

gemäß/according to § 19b Abs. 1 Chemikaliengesetz

Eine GLP-Inspektion zur Überwachung der Einhaltung der GLP-Grundsätze gemäß Chemikaliengesetz bzw. Richtlinie 2004/9/EG wurde durchgeführt in:

Assessment of conformity with GLP according to Chemikaliengesetz and Directive 2004/9/EC at:

Prüfeinrichtung / Test facility

[redacted]

Prüfung nach Kategorien / Areas of Expertise
(gemäß / according ChemVwV-GLP Nr. 5.3/OECD guidance)
1, 3, 4, 5, 6 , 8, 9 (toxikologische in Vitro Prüfungen an Säugerzellen und Bakterien)

Datum der Inspektion / Date of Inspection
(Tag.Monat.Jahr / day.month.year)
29. und 30. November 2010

Die genannte Prüfeinrichtung befindet sich im nationalen GLP-Überwachungsverfahren und wird regelmäßig auf Einhaltung der GLP-Grundsätze überwacht.

The above mentioned test facility is included in the national GLP Compliance Programme and is inspected on a regular basis.

Auf der Grundlage des Inspektionsberichtes wird hiermit bestätigt, dass in dieser Prüfeinrichtung die oben genannten Prüfungen unter Einhaltung der GLP-Grundsätze durchgeführt werden können.

Based on the inspection report it can be confirmed, that the test facility is able to conduct the aforementioned studies in compliance with the Principles of GLP.

Eine erneute behördliche Überprüfung der Einhaltung der GLP-Grundsätze durch die Prüfeinrichtung ist so rechtzeitig zu beantragen, dass die Folgeinspektion spätestens vier Jahre nach dem Beginn der o.g. Inspektion stattfinden kann. Ohne diesen Antrag wird die Prüfeinrichtung nach Ablauf der Frist aus dem deutschen GLP-Überwachungsprogramm genommen und diese GLP-Bescheinigung verliert ihre Gültigkeit.

Verification of the compliance of the test facility with the Principles of the GLP has to be applied for in time to allow for a follow-up inspection to take place within four years after commencing the above mentioned inspection. Elapsing this term, the test facility will be taken out of the German GLP-Monitoring Programme and this GLP Certificate becomes invalid.

Unterschrift, Datum / Signature, Date

Dr.-Ing. Stefan Hill - Präsident -
(Name und Funktion der verantwortlichen Person / name and function of responsible person)

Landesamt für Umwelt, Wasserwirtschaft und Gewerbeaufsicht
Kaiser-Friedrich-Straße 7, 55116 Mainz
(Name und Adresse der GLP-Überwachungsbehörde / Name and adress of the GLP Monitoring Authority)

MESSEN BEWERTEN BERATEN

16 ANNEX 2: MAIN STUDY - DETAILED DATA

16.1 Absorption Values Main Study

The absorption values were determined by photometric measurement. The values are presented in the following table:

Table 16.1-a Absorption Values Main Study

Nominal Concentration in mg/L	Absorption values			
	0 h	24 h	48 h	72 h
0	2	8	21	69
	2	8	24	95
	2	7	24	105
	2	8	31	101
	1	8	30	125
	1	8	30	98
1.0	3	9	40	109
	3	13	44	141
	4	10	40	162
3.2	3	8	31	124
	3	7	26	132
	2	8	36	140
10	3	7	24	51
	3	9	28	62
	3	7	27	60
32	3	6	10	13
	2	5	10	11
	4	7	10	12
100	3	8	10	10
	3	8	9	9
	4	9	9	9

For t = 0 h, the mean of the absorption values of the control was taken as reference; all other vessels were corrected by the respective deviation. For t = 24, 48, 72 h, the measured absorption values were corrected by the individual deviations of the replicates at t = 0 (measured absorption – mean absorption of controls at t = 0).

From the absorption values, cell numbers were calculated using the following equation:

$$y(t) = A + B * x(t)$$

with:

A	0
B	7574
y(t)	nominal number of cells at time t
x(t)	absorption at time t

The equation was calculated through microscopic measurement of the cell concentration and the absorption of seven different algal concentrations. The data were evaluated using linear fit.

16.2 Cell Numbers Main Study

The cell numbers for each replicate and the corresponding means and standard deviations are presented in the following table:

Table 16.2-a Cell Numbers Main Study

Nom. Conc. in mg/L	Repl.	Cell Number/mL			
		0 h	24 h	48 h	72 h
0	1	15148	60592	159054	522606
	2	15148	60592	181776	719530
	3	15148	53018	181776	795270
	4	15148	60592	234794	764974
	5	15148	68166	234794	954324
	6	15148	68166	234794	749826
	Mean	**15148**	**61854**	**204498**	**751088**
	SD	0	5702	34209	139033
1.0	1	15148	60592	295386	817992
	2	15148	90888	325682	1060360
	3	15148	60592	287812	1211840
	Mean	**15148**	**70691**	**302960**	**1030064**
	SD	0	17491	20039	198664
3.2	1	15148	53018	227220	931602
	2	15148	45444	189350	992194
	3	15148	60592	272664	1060360
	Mean	**15148**	**53018**	**229745**	**994719**
	SD	0	7574	41714	64416
10	1	15148	45444	174202	378700
	2	15148	60592	204498	462014
	3	15148	45444	196924	446866
	Mean	**15148**	**50493**	**191875**	**429193**
	SD	0	8746	15767	44380
32	1	15148	37870	68166	90888
	2	15148	37870	75740	83314
	3	15148	37870	60592	75740
	Mean	**15148**	**37870**	**68166**	**83314**
	SD	0	0	7574	7574
100	1	15148	53018	68166	68166
	2	15148	53018	60592	60592
	3	15148	53018	53018	53018
	Mean	**15148**	**53018**	**60592**	**60592**
	SD	0	0	7574	7574

SD = Standard Deviation

16.3 Growth Rate μ Main Study

The growth rate μ for each replicate and the corresponding means and standard deviations are given in the following table:

Table 16.3-a Growth Rate μ Main Study

Nom. Conc. in mg/L	Repl.	Growth Rate μ [day^{-1}]		
		24 h	48 h	72 h
0	1	1.39	1.18	1.18
	2	1.39	1.24	1.29
	3	1.25	1.24	1.32
	4	1.39	1.37	1.31
	5	1.50	1.37	1.38
	6	1.50	1.37	1.30
	Mean	**1.40**	**1.30**	**1.30**
	SD	0.09	0.09	0.07
1.0	1	1.39	1.49	1.33
	2	1.79	1.53	1.42
	3	1.39	1.47	1.46
	Mean	**1.52**	**1.50**	**1.40**
	SD	0.23	0.03	0.07
3.2	1	1.25	1.35	1.37
	2	1.10	1.26	1.39
	3	1.39	1.45	1.42
	Mean	**1.25**	**1.35**	**1.39**
	SD	0.14	0.09	0.02
10	1	1.10	1.22	1.07
	2	1.39	1.30	1.14
	3	1.10	1.28	1.13
	Mean	**1.19**	**1.27**	**1.11**
	SD	0.17	0.04	0.04
32	1	0.92	0.75	0.60
	2	0.92	0.80	0.57
	3	0.92	0.69	0.54
	Mean	**0.92**	**0.75**	**0.57**
	SD	0.00	0.06	0.03
100	1	1.25	0.75	0.50
	2	1.25	0.69	0.46
	3	1.25	0.63	0.42
	Mean	**1.25**	**0.69**	**0.46**
	SD	0.00	0.06	0.04

SD = Standard Deviation

16.4 Area under the Curve (Bio Mass Integral) Main Study

The Area under the Curve (Bio Mass Integral) AUC for each replicate and the corresponding means and standard deviations are given in the following table:

Table 16.4-a Area under the Curve (Bio Mass Integral) AUC Main Study

Nom. Conc. in mg/L	Repl.	AUC [Cell Concentration/mL*day]		
		24 h	48 h	72 h
0	1	22722	117397	443079
	2	22722	128758	564263
	3	18935	121184	594559
	4	22722	155267	640003
	5	26509	162841	742252
	6	26509	162841	640003
	Mean	**23353**	**141381**	**604027**
	SD	2851	21243	99267
1.0	1	22722	185563	727104
	2	37870	231007	908880
	3	22722	181776	916454
	Mean	**27771**	**199449**	**850813**
	SD	8746	27396	107202
3.2	1	18935	143906	708169
	2	15148	117397	693021
	3	22722	174202	825566
	Mean	**18935**	**145168**	**742252**
	SD	3787	28424	72548
10	1	15148	109823	371126
	2	22722	140119	458227
	3	15148	121184	427931
	Mean	**17673**	**123709**	**419095**
	SD	4373	15305	44218
32	1	11361	49231	113610
	2	11361	53018	117397
	3	11361	45444	98462
	Mean	**11361**	**49231**	**109823**
	SD	0	3787	10019
100	1	18935	64379	117397
	2	18935	60592	106036
	3	18935	56805	94675
	Mean	**18935**	**60592**	**106036**
	SD	0	3787	11361

SD = Standard Deviation

16.5 Yield Main Study

The Yield for each replicate and the corresponding means and standard deviations are given in the following table:

Table 16.5-a Yield Main Study

Nom. Conc. in mg/L	Repl.	Yield [Cell Concentration/mL]
		72 h
0	1	507458
	2	704382
	3	780122
	4	749826
	5	939176
	6	734678
	Mean	**735940**
	SD	139033
1.0	1	802844
	2	1045212
	3	1196692
	Mean	**1014916**
	SD	198664
3.2	1	916454
	2	977046
	3	1045212
	Mean	**979571**
	SD	64416
10	1	363552
	2	446866
	3	431718
	Mean	**414045**
	SD	44380
32	1	75740
	2	68166
	3	60592
	Mean	**68166**
	SD	7574
100	1	53018
	2	45444
	3	37870
	Mean	**45444**
	SD	7574

SD = Standard Deviation

16.6 Inhibition Values of Single Replicates Main Study

The inhibition values of the single replicates are presented in the following table:

Table 16.6-a Inhibition Values Main Study

Concentration (mg/L)	Replicate	Inhibition Growth Rate µ	Inhibition Area under the Curve AUC	Inhibition Yield
0	1	8.93	26.65	31.05
0	2	0.71	6.58	4.29
0	3	-1.87	1.57	-6.00
0	4	-0.87	-5.96	-1.89
0	5	-6.55	-22.88	-27.62
0	6	-0.35	-5.96	0.17
0	**Mean**	**0!**	**0!**	**0!**
0	SD	5.05	16.43	18.89
1.0		-2.59	-20.38	-9.09
1.0		-9.26	-50.47	-42.02
1.0		-12.70	-51.72	-62.61
1.0	**Mean**	**-8.18**	**-40.86**	**-37.91**
1.0	SD	5.14	17.75	26.99
3.2	1	-5.94	-17.24	-24.53
3.2	2	-7.56	-14.73	-32.76
3.2	3	-9.26	-36.68	-42.02
3.2	**Mean**	**-7.59**	**-22.88**	**-33.10**
3.2	SD	1.66	12.01	8.75
10	1	17.22	38.56	50.60
10	2	12.10	24.14	39.28
10	3	12.96	29.15	41.34
10	**Mean**	**14.09**	**30.62**	**43.74**
10	SD	2.74	7.32	6.03
32	1	53.92	81.19	89.71
32	2	56.16	80.56	90.74
32	3	58.61	83.70	91.77
32	**Mean**	**56.23**	**81.82**	**90.74**
32	SD	2.35	1.66	1.03
100	1	61.32	80.56	92.80
100	2	64.35	82.45	93.83
100	3	67.78	84.33	94.85
100	**Mean**	**64.48**	**82.45**	**93.83**
100	SD	3.23	1.88	1.03

17 ANNEX 3: REFERENCE STUDY - DETAILED DATA

17.1 Absorption Values Reference Study

The absorption values were determined by photometric measurement. The values are presented in the following table:

Table 17.1-a Absorption Values Reference Study

Nominal Concentration in mg/L	Absorption values			
	0 h	24 h	48 h	72 h
0	1	7	37	136
	1	7	40	138
	1	7	38	133
	2	8	31	106
	2	10	31	99
	2	10	37	147
0.046	1	7	31	113
	1	7	33	113
	2	7	31	109
0.063	1	7	33	111
	1	7	32	106
	2	7	30	109
0.1	2	8	30	101
	1	9	33	111
	2	9	31	104
0.16	1	8	36	118
	1	8	31	92
	1	7	32	90
0.25	1	8	27	83
	2	7	27	81
	2	8	28	87
0.4	1	10	22	56
	1	8	23	56
	2	7	25	59
0.63	3	7	14	27
	4	10	16	29
	2	6	14	26
1	3	7	10	11
	3	6	10	14
	4	8	10	13
1.6	6	9	10	12
	6	9	10	12
	5	7	7	9

For t = 0 h, the mean of the absorption values of the control was taken as reference; all other vessels were corrected by the respective deviation. For t = 24, 48, 72 h, the measured absorption values were corrected by the individual deviations of the replicates at t = 0 (measured absorption – mean absorption of controls at t = 0).

From the absorption values, cell numbers were calculated using the following equation:

$$y(t) = A + B * x(t)$$

with:

A	0
B	7574
y(t)	nominal number of cells at time t
x(t)	absorption at time t

The equation was calculated through microscopic measurement of the cell concentration and the absorption of seven different algal concentrations. The data were evaluated using linear fit.

17.2 Cell Numbers Reference Study

The cell numbers for each replicate and the corresponding means and standard deviations are presented in the following table:

Table 17.2-a Cell Numbers Reference Study

Nom. Conc. in mg/L	Repl.	Cell Number/mL			
		0 h	24 h	48 h	72 h
0	1	15148	60592	287812	1037638
	2	15148	60592	310534	1052786
	3	15148	60592	295386	1014916
	4	15148	60592	234794	802844
	5	15148	75740	234794	749826
	6	15148	75740	280238	1113378
	Mean	**15148**	**65641**	**273926**	**961898**
	SD	0	7822	31925	148342
0.046	1	15148	60592	242368	863436
	2	15148	60592	257516	863436
	3	15148	53018	234794	825566
	Mean	**15148**	**58067**	**244893**	**850813**
	SD	0	4373	11569	21864
0.063	1	15148	60592	257516	848288
	2	15148	60592	249942	810418
	3	15148	53018	227220	825566
	Mean	**15148**	**58067**	**244893**	**828091**
	SD	0	4373	15767	19061
0.10	1	15148	60592	227220	764974
	2	15148	75740	257516	848288
	3	15148	68166	234794	787696
	Mean	**15148**	**68166**	**239843**	**800319**
	SD	0	7574	15767	43068
0.16	1	15148	68166	280238	901306
	2	15148	68166	242368	704382
	3	15148	60592	249942	689234
	Mean	**15148**	**65641**	**257516**	**764974**
	SD	0	4373	20039	118310
0.25	1	15148	68166	212072	636216
	2	15148	53018	204498	613494
	3	15148	60592	212072	658938
	Mean	**15148**	**60592**	**209547**	**636216**
	SD	0	7574	4373	22722
0.4	1	15148	83314	174202	431718
	2	15148	68166	181776	431718
	3	15148	53018	189350	446866
	Mean	**15148**	**68166**	**181776**	**436767**
	SD	0	15148	7574	8746

Table 17.2-b Cell Numbers Reference Study (cont.)

Nom. Conc. in mg/L	Repl.	Cell Number/mL			
		0 h	**24 h**	**48 h**	**72 h**
0.63	1	15148	45444	98462	196924
	2	15148	60592	106036	204498
	3	15148	45444	106036	196924
	Mean	**15148**	**50493**	**103511**	**199449**
	SD	0	8746	4373	4373
1.0	1	15148	45444	68166	75740
	2	15148	37870	68166	98462
	3	15148	45444	60592	83314
	Mean	**15148**	**42919**	**65641**	**85839**
	SD	0	4373	4373	11569
1.6	1	15148	37870	45444	60592
	2	15148	37870	45444	60592
	3	15148	30296	30296	45444
	Mean	**15148**	**35345**	**40395**	**55543**
	SD	0	4373	8746	8746

SD = Standard Deviation

17.3 Growth Rate µ Reference Study

The growth rate µ for each replicate and the corresponding means and standard deviations are given in the following table:

Table 17.3-a Growth Rate µ Reference Study

Nom. Conc. in mg/L	Repl.	Growth Rate µ [day^{-1}]		
		24 h	48 h	72 h
0	1	1.39	1.47	1.41
	2	1.39	1.51	1.41
	3	1.39	1.49	1.40
	4	1.39	1.37	1.32
	5	1.61	1.37	1.30
	6	1.61	1.46	1.43
	Mean	**1.46**	**1.44**	**1.38**
	SD	0.12	0.06	0.05
0.046	1	1.39	1.39	1.35
	2	1.39	1.42	1.35
	3	1.25	1.37	1.33
	Mean	**1.34**	**1.39**	**1.34**
	SD	0.08	0.02	0.01
0.063	1	1.39	1.42	1.34
	2	1.39	1.40	1.33
	3	1.25	1.35	1.33
	Mean	**1.34**	**1.39**	**1.33**
	SD	0.08	0.03	0.01
0.10	1	1.39	1.35	1.31
	2	1.61	1.42	1.34
	3	1.50	1.37	1.32
	Mean	**1.50**	**1.38**	**1.32**
	SD	0.11	0.03	0.02
0.16	1	1.50	1.46	1.36
	2	1.50	1.39	1.28
	3	1.39	1.40	1.27
	Mean	**1.46**	**1.42**	**1.30**
	SD	0.07	0.04	0.05
0.25	1	1.50	1.32	1.25
	2	1.25	1.30	1.23
	3	1.39	1.32	1.26
	Mean	**1.38**	**1.31**	**1.25**
	SD	0.13	0.01	0.01
0.4	1	1.70	1.22	1.12
	2	1.50	1.24	1.12
	3	1.25	1.26	1.13
	Mean	**1.49**	**1.24**	**1.12**
	SD	0.23	0.02	0.01

Table 17.3-b Growth Rate μ Reference Study (cont.)

Nom. Conc. in mg/L	Repl.	Growth Rate μ [day^{-1}]		
		24 h	48 h	72 h
0.63	1	1.10	0.94	0.85
	2	1.39	0.97	0.87
	3	1.10	0.97	0.85
	Mean	**1.19**	**0.96**	**0.86**
	SD	0.17	0.02	0.01
1.0	1	1.10	0.75	0.54
	2	0.92	0.75	0.62
	3	1.10	0.69	0.57
	Mean	**1.04**	**0.73**	**0.58**
	SD	0.11	0.03	0.04
1.6	1	0.92	0.55	0.46
	2	0.92	0.55	0.46
	3	0.69	0.35	0.37
	Mean	**0.84**	**0.48**	**0.43**
	SD	0.13	0.12	0.06

SD = Standard Deviation

17.4 Area under the Curve (Bio Mass Integral) Reference Study

The Area under the Curve (Bio Mass Integral) AUC for each replicate and the corresponding means and standard deviations are given in the following table:

Table 17.4-a Area under the Curve (Bio Mass Integral) AUC Reference Study

Nom. Conc. in mg/L	Repl.	AUC [Cell Concentration/mL*day]		
		24 h	48 h	72 h
0	1	22722	181776	829353
	2	22722	193137	859649
	3	22722	185563	825566
	4	22722	155267	658938
	5	30296	170415	647577
	6	30296	193137	874797
	Mean	**25247**	**179883**	**782647**
	SD	3911	14716	101973
0.046	1	22722	159054	696808
	2	22722	166628	711956
	3	18935	147693	662725
	Mean	**21460**	**157792**	**690496**
	SD	2186	9530	25215
0.063	1	22722	166628	704382
	2	22722	162841	677873
	3	18935	143906	655151
	Mean	**21460**	**157792**	**679135**
	SD	2186	12174	24640
0.10	1	22722	151480	632429
	2	30296	181776	719530
	3	26509	162841	658938
	Mean	**26509**	**165366**	**670299**
	SD	3787	15305	44648
0.16	1	26509	185563	761187
	2	26509	166628	624855
	3	22722	162841	617281
	Mean	**25247**	**171677**	**667774**
	SD	2186	12174	80986
0.25	1	26509	151480	560476
	2	18935	132545	526393
	3	22722	143906	564263
	Mean	**22722**	**142644**	**550377**
	SD	3787	9530	20857
0.4	1	34083	147693	435505
	2	26509	136332	427931
	3	18935	124971	427931
	Mean	**26509**	**136332**	**430456**
	SD	7574	11361	4373

Table 17.4-b Area under the Curve (Bio Mass Integral) AUC Reference Study (cont.)

Nom. Conc. in mg/L	Repl.	AUC [Cell Concentration/mL*day]		
		24 h	**48 h**	**72 h**
0.63	1	15148	71953	204498
	2	22722	90888	231007
	3	15148	75740	212072
	Mean	**17673**	**79527**	**215859**
	SD	4373	10019	13654
1.0	1	15148	56805	113610
	2	11361	49231	117397
	3	15148	53018	109823
	Mean	**13886**	**53018**	**113610**
	SD	2186	3787	3787
1.6	1	11361	37870	75740
	2	11361	37870	75740
	3	7574	22722	45444
	Mean	**10099**	**32821**	**65641**
	SD	2186	8746	17491

SD = Standard Deviation

17.5 Yield Reference Study

The Yield for each replicate and the corresponding means and standard deviations are given in the following table:

Table 17.5-a Yield Reference Study

Nom. Conc. in mg/L	Repl.	Yield [Cell Concentration/mL]
		72 h
0	1	1022490
	2	1037638
	3	999768
	4	787696
	5	734678
	6	1098230
	Mean	**946750**
	SD	148342
0.046	1	848288
	2	848288
	3	810418
	Mean	**835665**
	SD	21864
0.063	1	833140
	2	795270
	3	810418
	Mean	**812943**
	SD	19061
0.10	1	749826
	2	833140
	3	772548
	Mean	**785171**
	SD	43068
0.16	1	886158
	2	689234
	3	674086
	Mean	**749826**
	SD	118310
0.25	1	621068
	2	598346
	3	643790
	Mean	**621068**
	SD	22722
0.4	1	416570
	2	416570
	3	431718
	Mean	**421619**
	SD	8746

Table 17.5-b Yield Reference Study (cont.)

Nom. Conc. in mg/L	Repl.	Yield [Cell Concentration/mL]
		72 h
0.63	1	181776
	2	189350
	3	181776
	Mean	**184301**
	SD	4373
1.0	1	60592
	2	83314
	3	68166
	Mean	**70691**
	SD	11569
1.6	1	45444
	2	45444
	3	30296
	Mean	**40395**
	SD	8746

SD = Standard Deviation

17.6 Inhibition Values of Single Replicates Reference Study

The inhibition values of the single replicates are presented in the following table:

Table 17.6-a Inhibition Values Reference Study

Concentration (mg/L)	Replicate	Inhibition Growth Rate μ	Inhibition Area under the Curve AUC	Inhibition Yield
0	1	-2.09	-5.97	-8.00
0	2	-2.44	-9.84	-9.60
0	3	-1.55	-5.48	-5.60
0	4	4.11	15.81	16.80
0	5	5.76	17.26	22.40
0	6	-3.79	-11.77	-16.00
0	**Mean**	**0!**	**0!**	**0!**
0	SD	3.93	13.03	15.67
0.04	1	2.35	10.97	10.40
0.04	2	2.35	9.03	10.40
0.04	3	3.43	15.32	14.40
0.04	**Mean**	**2.71**	**11.77**	**11.73**
0.04	SD	0.63	3.22	2.31
0.063	1	2.78	10.00	12.00
0.063	2	3.88	13.39	16.00
0.063	3	3.43	16.29	14.40
0.063	**Mean**	**3.36**	**13.23**	**14.13**
0.063	SD	0.55	3.15	2.01
0.1	1	5.28	19.19	20.80
0.1	2	2.78	8.06	12.00
0.1	3	4.57	15.81	18.40
0.1	**Mean**	**4.21**	**14.35**	**17.07**
0.1	SD	1.29	5.70	4.55
0.16	1	1.31	2.74	6.40
0.16	2	7.27	20.16	27.20
0.16	3	7.79	21.13	28.80
0.16	**Mean**	**5.46**	**14.68**	**20.80**
0.16	SD	3.60	10.35	12.50
0.25	1	9.73	28.39	34.40
0.25	2	10.61	32.74	36.80
0.25	3	8.88	27.90	32.00
0.25	**Mean**	**9.74**	**29.68**	**34.40**
0.25	SD	0.86	2.66	2.40

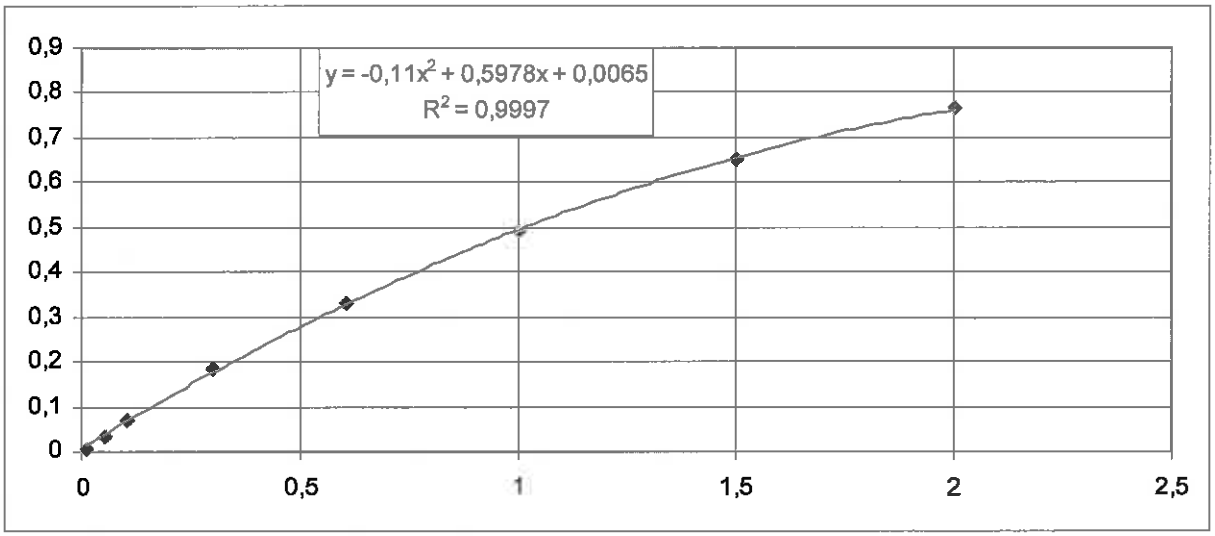
Table 17.6-b Inhibition Values Reference Study (cont.)

Concentration (mg/L)	Replicate	Inhibition Growth Rate µ	Inhibition Area under the Curve AUC	Inhibition Yield
0.4	1	38.05	44.35	56.00
0.4	2	37.14	45.32	56.00
0.4	3	38.05	45.32	54.40
0.4	**Mean**	**37.75**	**45.00**	**55.47**
0.4	SD	0.53	0.56	0.92
0.63	1	61.13	73.87	80.80
0.63	2	54.79	70.48	80.00
0.63	3	58.83	72.90	80.80
0.63	**Mean**	**58.25**	**72.42**	**80.53**
0.63	SD	3.21	1.74	0.46
1.0	1	66.52	85.48	93.60
1.0	2	66.52	85.00	91.20
1.0	3	73.47	85.97	92.80
1.0	**Mean**	**68.83**	**85.48**	**92.53**
1.0	SD	4.01	0.48	1.22
1.6	1	38.05	90.32	95.20
1.6	2	37.14	90.32	95.20
1.6	3	38.05	94.19	96.80
1.6	**Mean**	**37.75**	**91.61**	**95.73**
1.6	SD	0.53	2.23	0.92

18 ANNEX 4: CALIBRATION DATA [redacted]

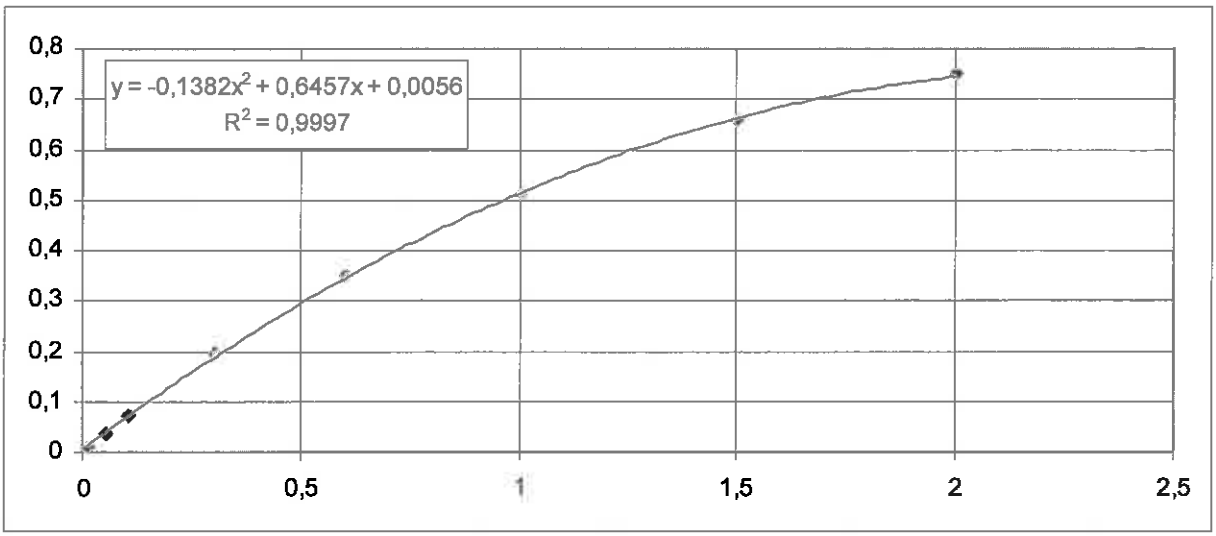
18.1 Calibration of [redacted]

Kal.-Std.	Absorption	SD (Abs.)	RSD in %
0.01 mg/L	0.00595	0.00012	1.9
0.05 mg/L	0.0333	0.0005	1.5
0.1 mg/L	0.06767	0.0006	0.9
0.3 mg/L	0.18384	0.00175	1
0.6 mg/L	0.33077	0.0023	0.7
1 mg/L	0.48946	0.00531	1.1
1.5 mg/L	0.65074	0.00723	1.1
2 mg/L	0.7626	0.00094	0.1



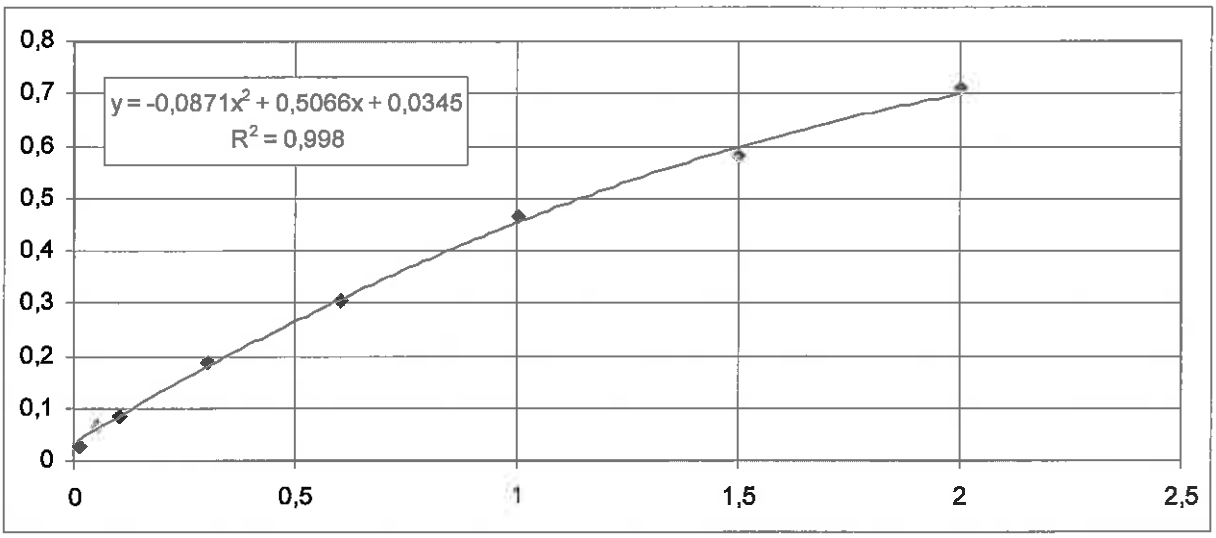
18.2 Calibration of [redacted]

Kal.-Std.	Absorption	SD (Abs.)	RSD in %
0.01 mg/L	0.00595	0.00032	5.3
0.05 mg/L	0.03439	0.0002	0.6
0.1 mg/L	0.07214	0.00081	1.1
0.3 mg/L	0.19303	0.00146	0.8
0.6 mg/L	0.34881	0.00666	1.9
1 mg/L	0.51058	0.00671	1.3
1.5 mg/L	0.656	0.00217	0.3



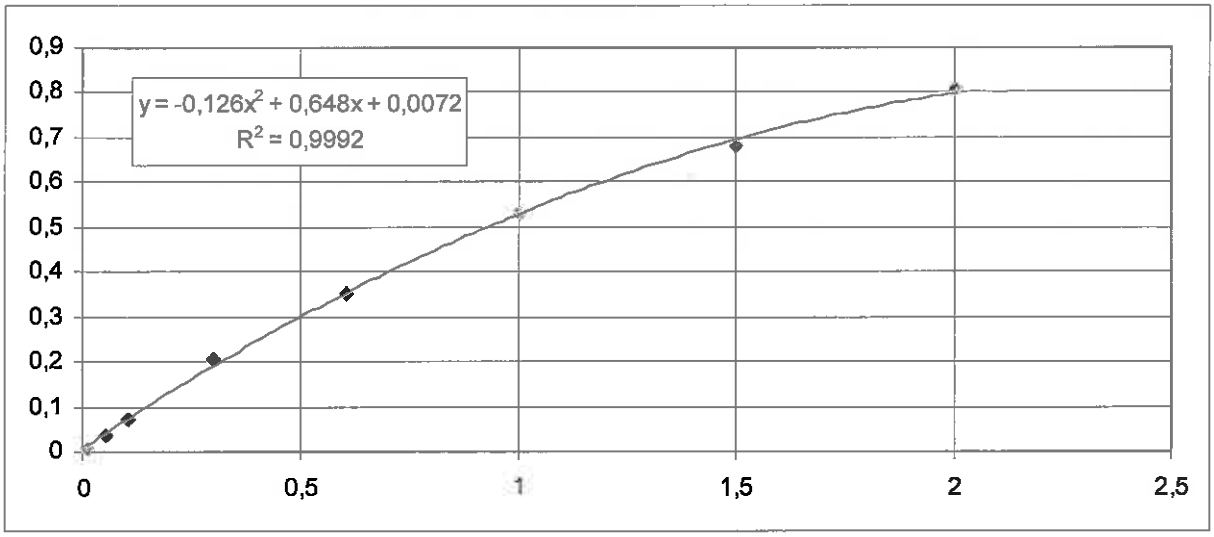
18.3 Calibration of [redacted]

Kal.-Std.	Absorption	SD (Abs.)	RSD in %
0.01 mg/L	0.02636	0.00066	2.5
0.05 mg/L	0.06585	0.00143	2.2
0.1 mg/L	0.08484	0.0016	1.9
0.3 mg/L	0.18689	0.00104	0.6
0.6 mg/L	0.30513	0.00874	2.9
1 mg/L	0.46624	0.01124	2.4
1.5 mg/L	0.58062	0.00083	0.1



18.4 Calibration of [redacted]

Kal.-Std.	Absorption	SD (Abs.)	RSD in %
0.01 mg/L	0.00555	0.00028	5.1
0.05 mg/L	0.0352	0.00013	0.4
0.1 mg/L	0.07199	0.00086	1.2
0.3 mg/L	0.204	0.00333	1.6
0.6 mg/L	0.35362	0.00216	0.6
1 mg/L	0.5307	0.00436	0.8
1.5 mg/L	0.68142	0.0053	0.8



Final Report Amendment No. 1

Original 2 **of** 2

Title of Study: Determination of 72h-EC50 of Safire 400 in *Desmodesmus subspicatus* according to OECD 201 resp. EU C.3

Study No.: [redacted]

Test Facility:

Study Director:

Final Report dated: [redacted]

1 AMENDMENT DESCRIPTION

In chapter 18.2 Calibration of [redacted] the following table

Kal.-Std.	Absorption	SD (Abs.)	RSD in %
0.01 mg/L	0.00595	0.00032	5.3
0.05 mg/L	0.03439	0.0002	0.6
0.1 mg/L	0.07214	0.00081	1.1
0.3 mg/L	0.19303	0.00146	0.8
0.6 mg/L	0.34881	0.00666	1.9
1 mg/L	0.51058	0.00671	1.3
1.5 mg/L	0.656	0.00217	0.3

is replaced by

Kal.-Std.	Absorption	SD (Abs.)	RSD in %
0.01 mg/L	0.00595	0.00032	5.3
0.05 mg/L	0.03439	0.00020	0.6
0.1 mg/L	0.07213	0.00081	1.1
0.3 mg/L	0.19304	0.00146	0.8
0.6 mg/L	0.34881	0.00666	1.9
1 mg/L	0.51058	0.00671	1.3
1.5 mg/L	0.65599	0.00217	0.3
2 mg/L	0.74838	0.00465	0.6

In chapter 18.3 Calibration of [redacted] the following table

Kal.-Std.	Absorption	SD (Abs.)	RSD in %
0.01 mg/L	0.02636	0.00066	2.5
0.05 mg/L	0.06585	0.00143	2.2
0.1 mg/L	0.08484	0.0016	1.9
0.3 mg/L	0.18689	0.00104	0.6
0.6 mg/L	0.30513	0.00874	2.9
1 mg/L	0.46624	0.01124	2.4
1.5 mg/L	0.58062	0.00083	0.1

Is replaced by

Kal.-Std.	Absorption	SD (Abs.)	RSD in %
0.01 mg/L	0.02636	0.00066	2.5
0.05 mg/L	0.06585	0.00143	2.2
0.1 mg/L	0.08484	0.00160	1.9
0.3 mg/L	0.18689	0.00104	0.6
0.6 mg/L	0.30513	0.00874	2.9
1 mg/L	0.46624	0.01124	2.4
1.5 mg/L	0.57825	0.00083	0.1
2 mg/L	0.70766	0.01647	2.3

In chapter 18.4 Calibration of [redacted] the following table

Kal.-Std.	Absorption	SD (Abs.)	RSD in %
0.01 mg/L	0.00555	0.00028	5.1
0.05 mg/L	0.0352	0.00013	0.4
0.1 mg/L	0.07199	0.00086	1.2
0.3 mg/L	0.204	0.00333	1.6
0.6 mg/L	0.35362	0.00216	0.6
1 mg/L	0.5307	0.00436	0.8
1.5 mg/L	0.68142	0.0053	0.8

is replaced by

Kal.-Std.	Absorption	SD (Abs.)	RSD in %
0.01 mg/L	0.00555	0.00028	5.1
0.05 mg/L	0.03520	0.00013	0.4
0.1 mg/L	0.07199	0.00086	1.2
0.3 mg/L	0.20400	0.00333	1.6
0.6 mg/L	0.35362	0.00216	0.6
1 mg/L	0.53070	0.00436	0.8
1.5 mg/L	0.68142	0.00530	0.8
2 mg/L	0.80617	0.01251	1.6

2 REASON FOR FINAL REPORT AMENDMENT

Error in Final Report (lines in tables omitted).

3 DISTRIBUTION OF FINAL REPORT AMENDMENT

Original 1 Archive of the test facility
Original 2 Sponsor

4 APPROVAL OF FINAL REPORT AMENDMENT:

[redacted]